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ORIGINAL ARTICLE

Genetic composition of Swiss and Austrian members of the apogamous *Dryopteris affinis* complex (Dryopteridaceae, Polypodiopsida) based on ISSR markers

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Abstract We have investigated genetic variability among 56 individuals of the apogamous *Dryopteris affinis* complex from 9 populations in Switzerland and Austria using inter-simple sequence repeats. Our sample included 6 diploids, 48 triploids, and 1 tetraploid and 1 pentaploid plant each. We found genetic differences between and within the different ploidy levels. All diploids genetically differed from each other. In contrast, among the triploid plants we only found seven different genotypes of which one was rather common. The tetraploid and pentaploid individuals were nested among the diploids and triploids. No hypotheses have yet been proposed to explain the origin of genetic variations such as those documented here. We hope that this study stimulates new ideas about the position and the genetic background of this taxonomically difficult species group, in particular, and of apogamous species complexes in ferns in general.

Keywords *Dryopteris affinis* · Apogamous fern species · Genetic variability · Different ploidy level · ISSR marker

Introduction

Apomixis has been of interest to botanists since the nineteenth century and a plethora of publications have been dedicated to it. A detailed review can be found in the book of Asker and Jeering (1992). Research on apomixis in different groups of flowering plants has recently again become of great interest (Grimanelli et al. 2001; Richards 2003; Hörandl et al. 2007) especially in connection with plant breeding (Hanna 1995; Grossniklaus et al. 2001; Savidan et al. 2001). With the aid of contemporary molecular methods it is now possible to obtain new insights into this phenomenon and to evaluate its significance for the evolution of plant diversity.

Apogamy in ferns was first described by De Bary (1878). Up to 10% of extant ferns species were assumed to show apogamous reproduction (Walker 1979). The term apogamy is mainly used for pteridophytes and refers to the formation of sporophytes from cells of unreduced gametophytes resulting from unreduced spores (diplospores) without any sexual process. In contrast with flowering plants, apogamy in ferns is usually obligate and the offspring of an apogamous individual is generally assumed to be genetically identical with the parent. Before meiosis takes place, eight spore mother cells with double the chromosome numbers of the sporophyte are formed as a result of endomitosis. So, unreduced spores (diplospores) result and during meiosis the identical chromosomes pair and, accordingly, the spores are genetically identical. The extent of formation of fertile spores, however, differs in correlation with ploidy level. The diploids and the triploids show a high percentage (ca. 80–90%) of regularly formed, fertile diplospores. The tetra and pentaploids form lower numbers of fertile diplospores (ca. 20–40%). The progeny of one apogamous plant is thought to be genetically identical. However, exceptions are mentioned, e.g., by Lin et al. (1992), Park and Kato (2005) and Ishikawa et al. (2003a, b) that assume that homologous pairing is a possible mechanism of formation of genetically different spores.

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Table 1 Locations of the populations and numbers of the plants studied

Location	Individuals	Numbers	Swiss coordinate	International coordinate	Altitude (m)
Küsnacht, Schmalzgrueb	12	1.1–1.3; 1.5–1.13	6882/2415	8°36′19″/47°19′07″	570
Maur, Forch, Guldenen	4	2.1–2.4	6922/24025	8°39′28″/47°18′24″	830
Einsiedeln; Gottschalkenberg 1	4	20.1–20.4	6907/22365	8°38′05″/47°09″27″	880
Einsiedeln; Gottschalkenberg 2	11	21.1–21.11	69125/2238	8°38′31″/47°09′32″	1,050
Einsiedeln; Gottschalkenberg 3	7	22.1–22.4	6912/22339	8°38′29″/47°09′19″	870
Ricken, Rittmarren	7	23.1–23.7	72234/23458	9°03′17″/47°15′03″	1,050
Ricken, Egg	5	24.1–24.5	7231/23415	9°03′53″/47°14′48″	1,180
Austria. Linz, Burk	2	16.1–16.2	–	48°11′53″/14°10′17″	600
Austria, Krimmlen	4	17.1–17.4	–	47°11′53″/12°10′17″	1,070

Assuming identical offspring, the process leading to genetically different apogamous lines would have to occur by repeated hybridization with closely related sexual species. If so, multiple origins of the apogamous species can be inferred. Different genotypes could also be the result of somatic mutations (Klekowski 1984). The occurrence of variant genotypes (genetically different clones) of apogamous ferns sometimes even within populations has been described by Watano and Iwatsuki (1988), Gastony and Windham (1989), Suzuki and Iwatsuki (1990), Lin et al. (1992, 1995), Takamiya et al. (2001), and Ishikawa et al. (2003a). Among European apogamous fern species, the triploid *Dryopteris remota* has been found to show populations with distinct genotypes (Schneller et al. 1998).

We here investigated different members of the *Dryopteris affinis* complex that has been shown to be apogamous (Schneller 1975; Bell 1992). *Dryopteris affinis* s.l. has been, and still is, problematic to taxonomists, resulting in many different and sometimes controversial, classifications (Oberholzer and Tavel 1937; Fraser-Jenkins 1980, 1996; Fraser-Jenkins and Reichstein 1984). Our main question was: does genetic variability occur within and/or among populations of plants with the same ploidy level or, as expected, only among different ploidy levels of *D. affinis*?

Materials and methods

Samples of *Dryopteris affinis* s.l. were collected in Switzerland and Austria in 2004, 2005 and 2007 (Table 1). Of each plant we collected an entire leaf for a herbarium sheet and two additional pinnae. Voucher specimens of all samples were deposited at the herbarium Zurich (Z). Immediately after collection one of the pinnae was used for flow cytometry. To identify the ploidy level we used a partec flow cytometer (Partec Ploidy Analyser PA1; Partec, Otto Hahn-Strasse 32, 48161, Münster, Germany). A small part of a fresh leaf was ground in 0.5 ml Partec HR-A solution with a razor blade. The assay was then filtered into a special test tube to which 2 ml Partec HR-B solution was added. The sample was then analyzed in the ploidy analyzer. For calibration, three plants of known ploidy level, one diploid one triploid, and one tetraploid were used.

Table 2 Sequence succession of the ISSR primers (Y = C, T; R = A, G)

UBC/Nr	Sequence of primers
827	ACA CAC ACA CAC ACA CG
834	AGA GAG AGA GAG AGA GYT
847	CAC ACA CAC ACA CAC ARC
855	ACA CAC ACA CAC ACA CYT
857	ACA CAC ACA CAC ACA CYG
861	ACA CAC ACA CAC ACA ACC

A pinna (or part of it) of each specimen was placed in a separate glass tube with a cap containing silica gel. The glass tubes were then stored at −20°C until part of the dried pinna was used for DNA extraction. For DNA extraction 0.015 g of dried leaf was ground and DNA was extracted using the DNeasy kit, following the manufacturer's instructions. The procedure used for the PCR reaction was the following: a mastermix for 16 tubes containing 464 µl ddH_2O, 48 µl $MgCl_2$, 80 µl Sigma PCR buffer P-2192, 128 µl peqGOLD dNTPs, 24 µl Primer, 5 µl Sigma taq DNA polymerase D-6677 was prepared. To each single probe (1/16th of mastermix) 2 µl of the fern extract was added. PCR reactions were run on a Techne TC 412 thermocycler with 4 min at 94°C, 35 cycles (1 min 94°C, 1 min 62°C, 2 min. 72°C, final extension 7 min 72°C and then the probes were kept at 10°C until they were used). The following primers gave interpretable results: UBC 827, 834, 847, 855, 857, 861 (Primer-Sets. http://www.michaelsmith.ubc.ca/sevices/NAPS/Primer_Sets/) (Table 2). PCR products were visualized by electrophoresis of agarose gels. We dissolved 1.1 g agarose in 75 ml TAE buffer by heating the solution to boiling

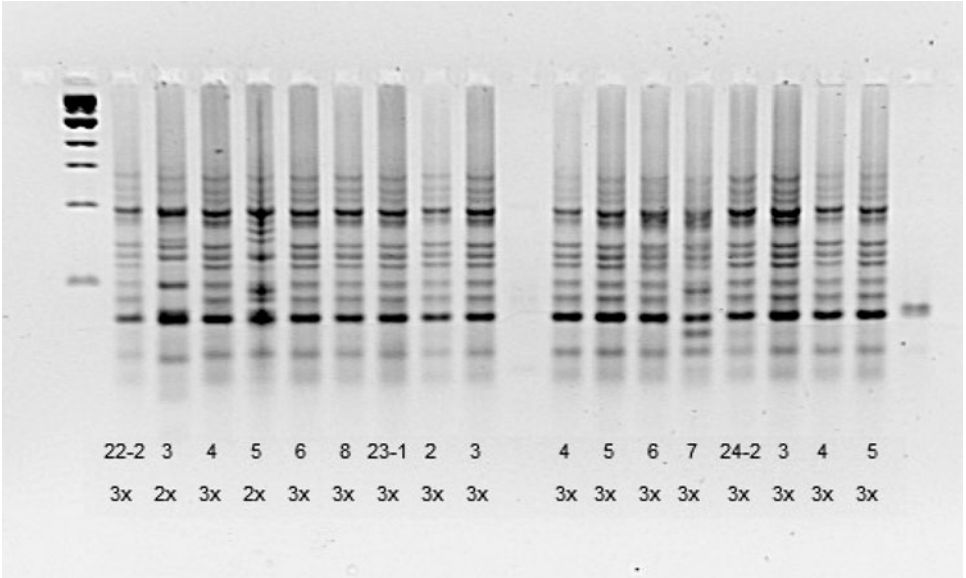

Fig. 1 ISSR phenogram of primer 857 showing different patterns of different plants indicated by collection numbers. No. 22-3, 22-5 are diploids, all the others are triploids, where 23-7 differs from the rest of the triploids, which show the same pattern. For comparison a 1 kb DNA ladder is shown at the *left side*

Table 3 Presence/absence matrix of the patterns resulting from the six primers

Population	Primer No.					
	827	834	847	855	857	861
a	000	0000	00000	0000	00000000	00000
b	000	0000	00000	0000	01000000	00000
c	000	1000	00000	0000	00000000	00000
d	000	0000	10000	0000	00000000	00000
e	000	0000	00000	0000	00000000	10000
f	000	0000	00000	0000	00100000	00100
g	100	0000	00000	0000	00000000	00000
h	100	0000	00000	0100	00010000	00000
i	100	0100	00000	0000	00000000	00000
k	010	0100	00000	0100	10000000	00000
l	010	1000	01000	0100	10000000	00100
m	010	0100	00100	0100	00000000	01000
n	010	0010	00010	0010	00000100	00010
o	100	0100	00100	0001	00001000	00000
p	001	0100	10000	1000	00000010	10000
q	000	0001	00001	0000	10000000	00001
r	000	1000	01000	0000	00000001	00000

Seventeen phenotypes of different plants and different ploidy level were found

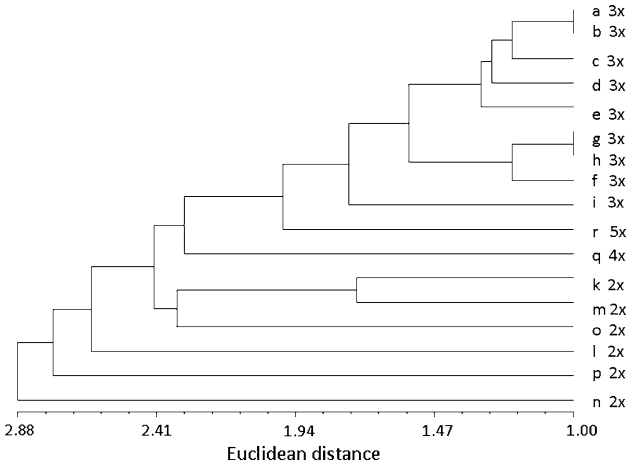

Fig. 2 UPGM tree based on calculation of Euclidean similarity using the presence/absence matrix. The *letters* correspond to the letter of ISSR phenotypes shown in Tables 3 and 4. Ploidy levels are indicated. Note the substantial difference within the diploids

point. After cooling to 50°C, 1.2 µl ethidium bromide was added, mixed with the gel, and the gel was poured into the electrophoresis chamber (Bio RAD) and a comb (20 teeth) was added. After about 30 min., the gel was cool enough to load 15 µl probe with 5 µl loading buffer per track. The size of the product was evaluated using 10 µl ladder. The gel was run for 3 h at 40 V. The bands were developed under UV light and photographs were taken (Fig. 1). Banding patterns, considering only clearly distinct bands, were visually scored for a presence/absence matrix (Table 3). A similarity matrix (ean distance) based on polymorphic markers only was calculated and an UPGMA tree was created (Rohlf 2000) (Table 3; Fig. 2). When using the Nei (1978) boostrapped distance measure (Miller 2000) based on the banding pattern 1 for absent and 2 for present a very similar tree was calculated (not shown here).

One diploid (20.1, Table 4), one triploid (20.3, Table 4), and one tetraploid plant (21.6, Table 4) were cytologically investigated using the acetocarmine technique (Manton 1950). These plants were then used as standards to compare

Table 4 Distribution of the ISSR phenotypes within and among the populations

Location	No. of plants	Collection	ISSR phenotype	Ploidy
Einsiedeln, Gottschalkenberg 1	1	20.1	l	2*x*
	2	20.2	r	5*x*
	3	20.3	a	3*x*
	4	20.4	e	3*x*
Einsiedeln, Gottschalkenberg 2	5	21.1	o	2*x*
	6	21.2	a	3*x*
	7	21.3	k	2*x*
	8	21.4	a	3*x*
	9	21.5	h	3*x*
	10	21.6	q	4*x*
	11	21.7	a	3*x*
	12	21.8	a	3*x*
	13	21.9	a	3*x*
	14	21.10	m	2*x*
	15	21.11	c	3*x*
Einsiedeln, Gottschalkenberg 3	16	22.1	d	3*x*
	17	22.2	a	3*x*
	18	22.3	p	2*x*
	19	22.4	a	3*x*
	20	22.5	n	2*x*
	21	22.6	a	3*x*
	22	22.8	a	3*x*
Ricken, Rittmarren	23	23.1	a	3*x*
	24	23.2	a	3*x*
	25	23.3	a	3*x*
	26	23.4	f	3*x*
	27	23.5	a	3*x*
	28	23.6	a	3*x*
	29	23.7	i	3*x*
Ricken, Regelstein	30	24.1	a	3*x*
	31	24.2	e	3*x*
	32	24.3	a	3*x*
	33	24.4	a	3*x*
	34	24.5	g	3*x*
Küsnacht, Schmalzgrueb	35	1.1	a	3*x*
	36	1.2	a	3*x*
	37	1.3	a	3*x*
	38	1.5	a	3*x*
	39	1.6	a	3*x*
	40	1.7	a	3*x*
	41	1.8	a	3*x*
	42	1.9	a	3*x*
	43	1.10	b	3*x*
	44	1.11	a	3*x*
	45	1.12	a	3*x*
	46	1.13	b	3*x*
Forch, Guldenen	47	2.1	a	3*x*
	48	2.2	a	3*x*
	49	2.3	a	3*x*
	50	2.4	a	3*x*
Österreich, Burk, Linz	51	16.1	a	3*x*
	52	16.2	a	3*x*
Österreich, Burk, Linz	53	17.1	a	3*x*
	54	17.2	a	3*x*
	55	17.3	a	3*x*
	56	17.4	a	3*x*

the other samples by flow cytometry (Watson 1991; Shapiro 2003).

Results

Among the 56 accessions, the flow cytometry analysis revealed DNA amounts corresponding to diploids in 6 cases, to triploids in 48 cases, and to one tetraploid and pentaploid.

The PCR of the six primers (Table 3) exhibited 17 different genotypes. The six diploid plants each showed a unique phenotype bands differing from the others in two to five loci (Table 3). In contrast, among the triploids 38 of 48 plants shared the same genotype (Table 4). The remaining triploid plants included six unique phenotypes differing in one or in two bands from each other. Two unique genotypes were also found in the tetraploid and pentaploid plants. Within the populations, Gottschalkenberg (all three sites) had five unique bands, the populations from Ricken (Rittmarren/Egg) five (plus two shared with Gottschalkenberg), the population from Küsnacht two, while Forch, Burk, and Kimmlen only had one unique band each. The UPGMA analysis grouped all diploid plants in one cluster and all the triploids in another cluster (Fig. 2). The tetraploid and pentaploid samples were placed between the diploids and the triploids. The pentaploid individual was more similar to the triploids than to the diploids.

Discussion

Studies on genetically distinguishable individuals within or among populations are known for several apogamous, homosporous fern species (Suzuki and Iwatsuki 1990; Lin et al. 1995; Takamiya et al. 2001; Ishikawa et al. 2003a, b). The number of variants within a species differed in these

studies, but this partly depended on the number of plants per species investigated and the geographic distances between sampled populations. Previous studies have shown that variants within a ploidy level not only occur between populations, which one would expect, but also within populations (Suzuki and Iwatsuki 1990; Lin et al. 1995; Takamiya et al. 2001; Ishikawa et al. 2003a, b). This was also the case in our study. Our 56 samples (9 populations) of *D. affinis* included 48 triploids involving 7 unique phenotypes. All of these phenotypes were more similar to each other than to the phenotypes of the diploid taxon (Fig. 2). The genetic variability of the triploids within population varied from none to two. In contrast, we were surprised by the striking differences within the diploid accessions, of which each individual showed a different ISSR-phenotype. Connecting this variability with the occurrence of somatic mutations, we could argue that diploids have already been present for a long time-span and that they are older than the triploids, because the amount of somatic mutations increases with time (Klekowski 1988). Schneller et al. (1998) assumed that the genetic diversity of triploid apogamous *Dryoperis remota* is related to somatic mutations.

In apogamous fern species one would expect to find the same genotype among many individuals within a given population, because the spores of one plant are thought to be genetically identical. In our study, this was not the case in the diploid specimens as far as can be interpreted from only six individuals. In the triploid cluster a common phenotype was found within and among all the populations, which suggests recent colonization through spores of the same "clone" within and among even distant populations. The diploids were less frequent within populations and appeared to produce new individuals within a population only rarely. They could be also interpreted as the result of multiple origins based on different crossing processes.

Another, controversial, explanation for the different genotypes could be the assumed presence of homologous pairing in meiosis (Klekowski 1973) which would lead to segregation in offspring. There are further arguments based on the findings of Lin et al. (1992) for *Dryopteris pacifica* and Ishikawa et al. (2003a, b) for *Dryopteris nippoensis* that triploids would be able to reduce their chromosome numbers by forming pairs and univalents in 16 spore mother cells which could then rarely lead to viable apogamous diploid spores. Such spores would form diploid prothalli and develop either into apogamous, diploid sporophytes or they could hybridize with diploid sexual plants to lead again to triploid apogamous plants. Because in our study the triploids were less variable and one genotype was found across all the populations the latter processes seem to be, if they really occur, unlikely.

One interpretation would be that triploids are the product of a crossing between diploid apogamous and diploid sexual members of *Dryopteris filix-mas* s.l. The diploid sexual parents of *D. filix-mas* s.l. (*D. oreades* and *D. caucasica*) are not, or not any more, found within the distribution area of the investigated samples. This hypothesis is confirmed by similarity of morphological characters in *D. affinis* and *D. filix-mas* in general. In addition, the dark blue spot at the base of the pinnae, which is present in the diploid *D. affinis*, is found also in all the other apogamous taxa of *D. affinis* s.l.

The tetraploid and pentaploid members have to be assumed to be of recent origin and could have been formed within the populations as the result of crossings between the tetraploid *D. filix-mas* ($2n = 4x = 164$) and the diploid ($2n = 2x = 82$) or triploid *D. affinis* ($2n = 3x = 123$). This is in accordance with the position of these "hybrid" plants between the diploids and the triploids in the UPGMA tree.

Our results lead to many still unanswered questions. For example, are the diploid individuals introduced more or less randomly from genetically distinct source populations? Does the observed genetic variability of the diploids relate to somatic mutation or is it linked to multiple origins of diploid aposporous taxa? Are the triploids of hybrid origin related to the diploid taxon or a product of them? Is the variability of the triploids only a product of somatic mutations? It is obvious that the triploids have not been produced by hybridization at the site they are currently found. Are the diploids and possibly also the triploids aposporous lineages of rather old origin?

In conclusion, we still do not know which processes led to the genetic variability of the diploid and triploid members found in this study and we do not know when and where these taxa originated. We believe that the age of the aposporous members is an important factor for the observed present conditions.

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